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14. ABSTRACT

The overall purpose of this project is to develop targetable water-soluble polymer-antiangiogenic drug conjugates for systemic breast cancer therapy. The rationale is that by selectively targeting polymer-antiangiogenic drug conjugates to alpha-v-beta-3 receptors on endothelial cells of tumor neovasculature; it is possible to restrict the biodistribution of the antiangiogenic drug to the vascular space, thereby increasing tumor accumulation and subsequent antitumor potency of the drug and decreasing dose-limiting toxicity. In year 1 the following were accomplished: i) Synthesis of a series of targetable HPMA copolymer-antiangiogenic drug conjugates; ii) Physicochemical characterization of the synthesized conjugates. In year 2 (no cost extension period the following wasaccomplished: iii) In vitro evaluation of targeting efficacy of the synthesized conjugates against model endothelial cell line and iv) In vitro evaluation of antiproliferative efficacy of the synthesized conjugates against the same cell line. Additionally synthetic strategies were established to modify the drug with a lysosomally degradable spacer. Results demonstrate the feasibility of synthesizing angiogenesis targetable HPMA copolymer drug conjugates and their potential in specifically binding to endothelial cell surface receptors as well as causing inhibition of cell proliferation. Overall these conjugates have the potential to treat breast cancer by inhibiting the angiogenic vasculature around the tumor.

15. SUBJECT TERMS
Targeted drug delivery, polymers, antiangiogenic, breast cancer, water-soluble

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Table of Contents

INTRODUCTION:

The **overall purpose** of this project is to develop targetable water-soluble polymer-antiangiogenic drug conjugates for systemic breast cancer therapy. The **rationale** is that by selectively targeting polymer-antiangiogenic drug conjugates to alpha-v-beta-3 receptors on endothelial cells of tumor neovasculature; it is possible to restrict the biodistribution of the antiangiogenic drug to the vascular space, thereby increasing tumor accumulation and subsequent antitumor potency of the drug and decreasing dose-limiting toxicity. Three Specific Aims were proposed:

1) To synthesize and physicochemically characterize *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-TNP-470 conjugates with and without the targeting moiety (RGD4C) in the side chains.

2) To evaluate the targeting/binding efficacy of the conjugates on a model endothelial cell line *in vitro*.

3) To evaluate the antiproliferative efficacy of the conjugates in the model endothelial cell line *in vitro*.

All of the specific aims were accomplished at the conclusion of the second year (no cost extension period). Results of accomplishments in Year 2 are described below and a manuscript is under preparation. For details of experiments accomplished in Year 1 please see Year 1 report and its appendices.

BODY:

A) Synthesis of HPMA copolymer-antiangiogenic drug conjugates with and without the targeting moiety (RGD4C) in the side chains:

For this portion of the project the following tasks and subtasks were proposed:

Task 1. Synthesis of the polymer-drug-targeting moiety conjugates.

a. Synthesis of monomeric units, and characterization by mass spectrometry.

b. Synthesis of polymer precursors of HPMA copolymers containing reactive functional groups by free-radical precipitation copolymerization and characterization of reactive group content by UV spectrophotometry.

c. Conjugation of TNP-470 and RGD4C to HPMA copolymer precursors by consecutive aminolysis reaction.

d. Purification of the conjugates by size-exclusion chromatography followed by dialysis and lyophilization.

We successfully completed Task 1 in Year 1 and disseminated the results in the annual Era of Hope meeting 2005 in Philadelphia, PA (See Year 1 report).

A glycine derivative of Fumagillin (Fumagillol-Glycine, Fu-Gly) was used as a model drug instead of TNP-470 to permit easy attachment of the drug to the polymer backbone. Fumagillin derivatives such as Fu-Gly also have known antiangiogenic effects like the proposed drug TNP-470 which is a derivative of the parent compound Fumagillin. The purpose of using Fu-Gly was to demonstrate proof of concept which can then be extended to other model antiangiogenic drugs including TNP-470.

The following conjugates were successfully synthesized. Details of synthesis and characterization are provided in Appendix 1 of Year 1 report.

P1: HPMA homopolymer (Control polymer with no drug or targeting moiety)
P2: HPMA copolymer-(GG-RGD4C)-GFLGG-Fu (Polymer with drug and targeting moiety)
P3: HPMA copolymer-(GG-RGD4C) (Polymer with targeting moiety but no drug)
P4: HPMA copolymer-GFLGG-Fu (Polymer with drug but no targeting moiety)

B) Physiochemical characterization of synthesized conjugates:

For this portion of the project the following tasks and subtasks were proposed:

Task 2. Physicochemical characterization of the conjugates.

a. Drug content measurement by UV spectrophotometry.
b. Targeting moiety content measurement by amino acid analysis.
c. Molecular weight and polydispersity measurement by size-exclusion chromatography.

We successfully completed Task 2 and partially disseminated the results in the annual Era of Hope meeting in Philadelphia, PA (See Appendix 1 (Abstract) and 2 (poster) of Year 1 report). The complete physicochemical characterization data is tabulated below (Table 1):

Table 1. Physicochemical properties of targetable HPMA-antiangiogenic drug conjugates

Sample	Description	Molar feed ratio			ONp content (mmole/g P)	RG**D**4C content (mmole/g P)	Fu content (mmole/g P)	Mw (kDa)	Mw/Mn
		HPMA	GG-ONp	GFLGG-Fu					
P1	HPMA	100	0	0	-	-	-	123.0	1.8
P2	HPMA-(GG-RG**D**4C)-GFLGG-Fu	85	10	5	0.5	0.17	0.28	132.0	1.9
P3	HPMA-(GG-RG**D**4C)	90	10	0	0.6	0.24	-	38.1	1.4
P4	HPMA-GFLGG-Fu	95	0	5	-	-	0.24	83.5	1.7

Legends:
HPMA= N-(2-hydroxypropyl) methacrylamide, G=Glycine, F=Phenylalanine, L=Leucine, Fu=Fumagillol, M_w=weight average molecular weight, M_n=number average molecular weight, Mw/Mn=polydispersity

The synthesized conjugates were successfully characterized. The contents of drug and targeting moiety were proportional to their molar feed ratios and the data was consistent with report of similar systems in the literature.

C) In vitro evaluation of synthesized conjugates:

For this portion of the project the following tasks and subtasks were proposed:

Task 3. In vitro evaluation of targeting efficiency, and antiproliferative activity of the conjugates.

a. Endothelial cell adhesion assay to determine binding/targeting affinity of conjugates.
b. Endothelial cell growth inhibition assay to determine the efficacy of the conjugates.

The synthesized copolymer conjugates (Table 1) were successfully evaluated for their in vitro binding efficacy against the Human Umbilical Vein Endothelial Cell (HUVEC) line. The effect of the synthesized conjugates on the growth inhibition of HUVECs was evaluated in vitro using a standard MTT assay.

One challenge with the polymer-drug conjugate synthesis that was encountered in Year 1 was that the presence of Gly in Fu-Gly resulted in a polymer conjugate with the drug attached via a glycylphenylalanylleucylglycylglycine (GFLG**G**) spacer rather than the well established lysosomally degradable (GFLG) (one less glycine) spacer. It was anticipated that the biodegradability and release profile of the drug from the GFLGG spacer might be somewhat different than the GFLG spacer. As an alternative, we had proposed to establish a synthetic strategy to make a polymer conjugate of the drug with a GFLG spacer. To this regard comonomers were successfully synthesized and characterized demonstrating the feasibility of synthesizing polymer conjugates with the GFLG spacer.

Please see Appendix 1 for details of the methodology and results of in vitro evaluations and new synthesis and characterizations of comonomers as outlined in Task 3.

KEY RESEARCH ACCOMPLISHMENTS:

1) Synthesis of targetable polymer-antiangiogenic drug conjugates for systemic breast cancer therapy.

2) Physicochemical characterization of synthesized conjugates.

3) In vitro evaluation of binding efficacy of synthesized conjugates.

4) In vitro evaluation of antiproliferative efficacy of synthesized conjugates against model endothelial cell line.

5) Additional work demonstrating successful synthesis and characterization of antiangiogenic drug bearing comonomers with GFLG spacer.

6) Presented and partially disseminated research findings in the 2005 Era of Hope Meeting.

7) Participated in scientific meetings related to the area of controlled drug delivery:
   - Controlled Release Society Meeting (CRS-June 2005, Miami, Florida).
   - 12th International Symposium on Recent Advances in Drug Delivery Systems (February, 2005, Salt Lake City, Utah).

REPORTABLE OUTCOMES:

1) Abstract and poster presentation in Era of Hope Meeting (2005).

2) Manuscript under preparation.

3) Training opportunities: Jun Lee, a graduate research assistant who was a Pharm D candidate, was trained and successfully developed methodologies for synthesis and characterization of the second series of comonomers. He currently enrolled in the Pharm D/ PhD graduate program and plans to pursue research on targeted antiangiogenic therapy using polymeric conjugates.

CONCLUSIONS:

In summary progress was made in all of the specific aims. Some challenges in the synthetic methodology were addressed successfully by establishing alternative strategies to optimize the spacer between the drug and the polymer backbone. In the no cost extension period (Year 2), the ongoing in vitro evaluations were successfully completed. This work provides a platform for subsequent development of angiogenesis targeted polymeric nanoconstructs for improving the treatment regime of breast cancer. Moreover the general concept of angiogenic targeting can be extended to other cancer related malignancies which can have a profound impact on the elimination of cancer related deaths worldwide.

APPENDICES:

Appendix 1: Supplemental data describing details of experimental methods and results of studies accomplished in Year 2 beyond what was reported in the report of Year 1.

Appendix 1: Supplemental data

Legends of Polymer drug conjugates referred in text below:
P1: HPMA
P2: HPMA-(GG-RGD4C)-GFLGG-Fu
P3: HPMA-(GG-RGD4C)
P4: HPMA-GFLGG-Fu

1. In vitro evaluation of binding efficacy of HPMA-antiangiogenic drug conjugates
The bioactivity of the RGD peptide after polymer conjugation was assessed using an inhibition assay as previously described (1). Briefly, flat bottom 96-well culture plates (Corning Inc., NY, USA) were coated with 0.5 µg/well fibrinogen overnight at 4°C. After three rinses with phosphate buffered saline (PBS), the wells were blocked with 3% bovine serum albumin (BSA) for 1 hour at 37°C and washed three times with PBS. Trypsinized HUVECs were resuspended in serum free media and, in separate experiments, were incubated (15 min at 22°C) with 1) HPMA copolymer-RGD4C conjugates and appropriate controls (namely compounds P1-P4) and free RGD4C. The treated HUVECs were plated at a density of 30,000 cells/well and allowed to attach for 1 hour at 37°C. After incubation the unattached cells were removed by rinsing the wells with PBS. The attached cells were fixed with 3.7% formaldehyde, stained with 0.5% crystal violet and assayed at 540 nm on a microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA, USA). Student's two-tailed unpaired t-test was used to assess the differences of cell adhesion between the polymers. The results of the bioadhesion assay are given in Fig 1.

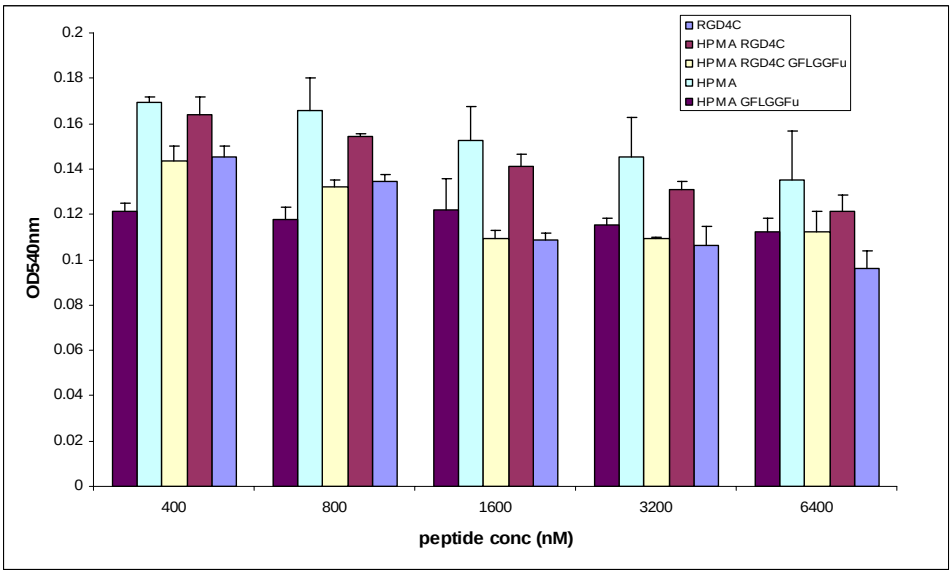

Figure 1 Endothelial cell adhesion assay with HPMA copolymers and peptides. The $\alpha_V\beta_3$ mediated adhesion of HUVECs to fibrinogen is inhibited on exposure of HUVECs to HPMA copolymer-RGD4C conjugates and free RGD4C. The values represent means of triplicate ± SD.

The results show HPMA-RGD4C conjugate without drug attached (P3) showed inhibition of bioadhesion with increasing concentration of peptide. This demonstrates the ability of the conjugates to bind to $\alpha_V\beta_3$ on endothelial cells. However the binding inhibition of the free peptide RGD4C was higher than the polymeric conjugate indicating a reduction of bioadhesion upon polymer conjugation of the peptide. Control conjugate HPMA homopolymer (P1) with no targeting caused significantly less inhibition of binding. However at higher concentrations some binding inhibition was observed possibly due to non-specific interactions of the polymer. Finally HPMA conjugates of the antiangiogenic drug Fu-Gly with or without targeting moiety (P2 and P4) showed inhibition of binding as well. However this effect was not fully explainable and possibly could be due to an inhibitory effect of the drug itself on the HUVEC cells. Overall RGD4C conjugated to the polymer demonstrated the ability to retain binding properties which will be of significance in subsequent in vivo evaluations of targetability to tumor angiogenesis.

(See next page for continuation)

2. In vitro evaluation of antiproliferative action of HPMA-antiangiogenic drug conjugates

The purpose here was to evaluate whether the conjugates containing the drug Fu-Gly are bioactive. Proliferation of normally quiescent endothelial cells is a critical step required for angiogenesis. The antiproliferative action of the polymer-Fu-Gly conjugates was therefore be evaluated on HUVECs to determine their relative biological activity in comparison to the free drug. Briefly HUVEC cells from confluent cultures were seeded at a density of 1,000 (100 µl) cells per well in 96-well flat-bottomed microtiter plate and incubated at 37°C for 24h. 100 µl solutions of free drug and different HPMA conjugates with and without drug and/or targeting moiety (P1-P4) were added to the wells over a range of concentration (drug equivalent). Plates were incubated at 37°C for 3 days. Cell proliferation was quantified by using the standard MTT Assay. Briefly MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was reduced by mitochondrial dehydrogenase enzyme from viable cells to form dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilization of the cells by the addition of DMSO resulted in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product created. The color was quantified using a simple colorimetric assay at 560nm. The inhibitory ratio of the test compound was calculated according to the following formula: inhibition ratio (%) = 100 x (C-T)/C, where T is an absorbance of well containing a test compound and C is an absorbance of well containing no test compound (control). Results are given in Fig 2.

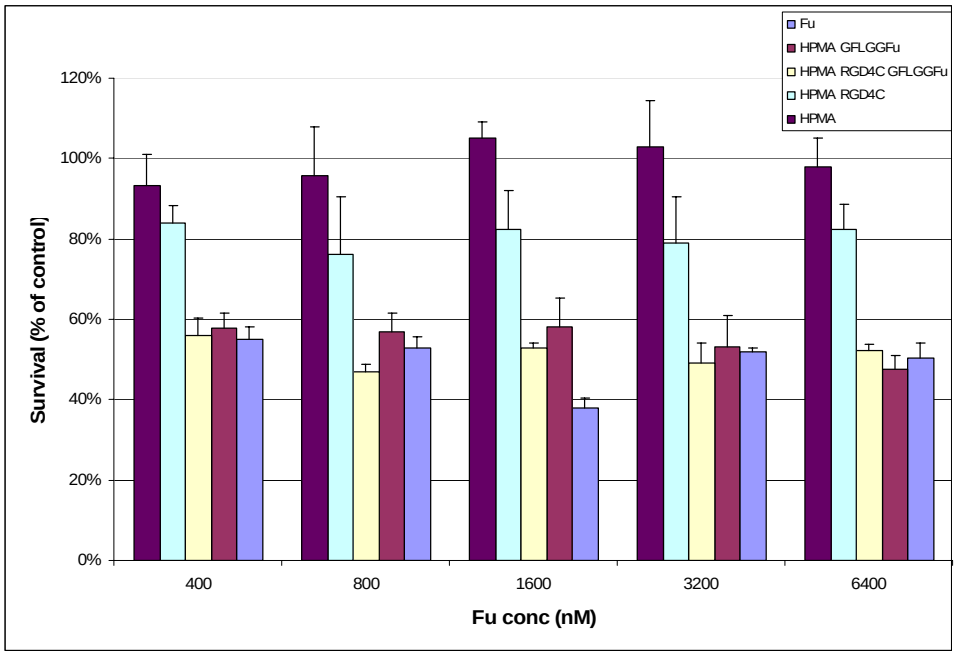

Figure 2 MTT growth inhibition assay of HPMA copolymers conjugates against HUVEC cells. The values represent means of quadruplet ± SD.

Results show HPMA-RGD4C-Fu-Gly conjugates with and without RGD4C (P2 and P4) showed a cytostatic effect on the HUVECs at all the concentrations studied. This was comparable to that of free Fu-Gly demonstrating that the targetable polymeric conjugate is active. HPMA homopolymer (P1) showed almost no effect on cell proliferation as expected demonstrating that the polymer backbone by itself is biocompatible. HPMA-RGD4C control conjugate with no drug attached showed some degree of cytostatic effect however this effect was significantly less than the drug containing conjugates. A limited degree of effect could be possibly due to the effect of RGD4C by itself however detailed studies are needed to understand this mechanism. Overall the results demonstrate that polymeric conjugates of the antiangiogenic drug Fu-Gly are bioactive.

(See next page for continuation)

3. Synthesis and characterization of a polymerizable Fumagillin derivative with Gly-Phe-Leu-Gly spacer

The synthetic strategy has been schematically represented in Fig 3. The details of the methodology are given in Fig 3 below:

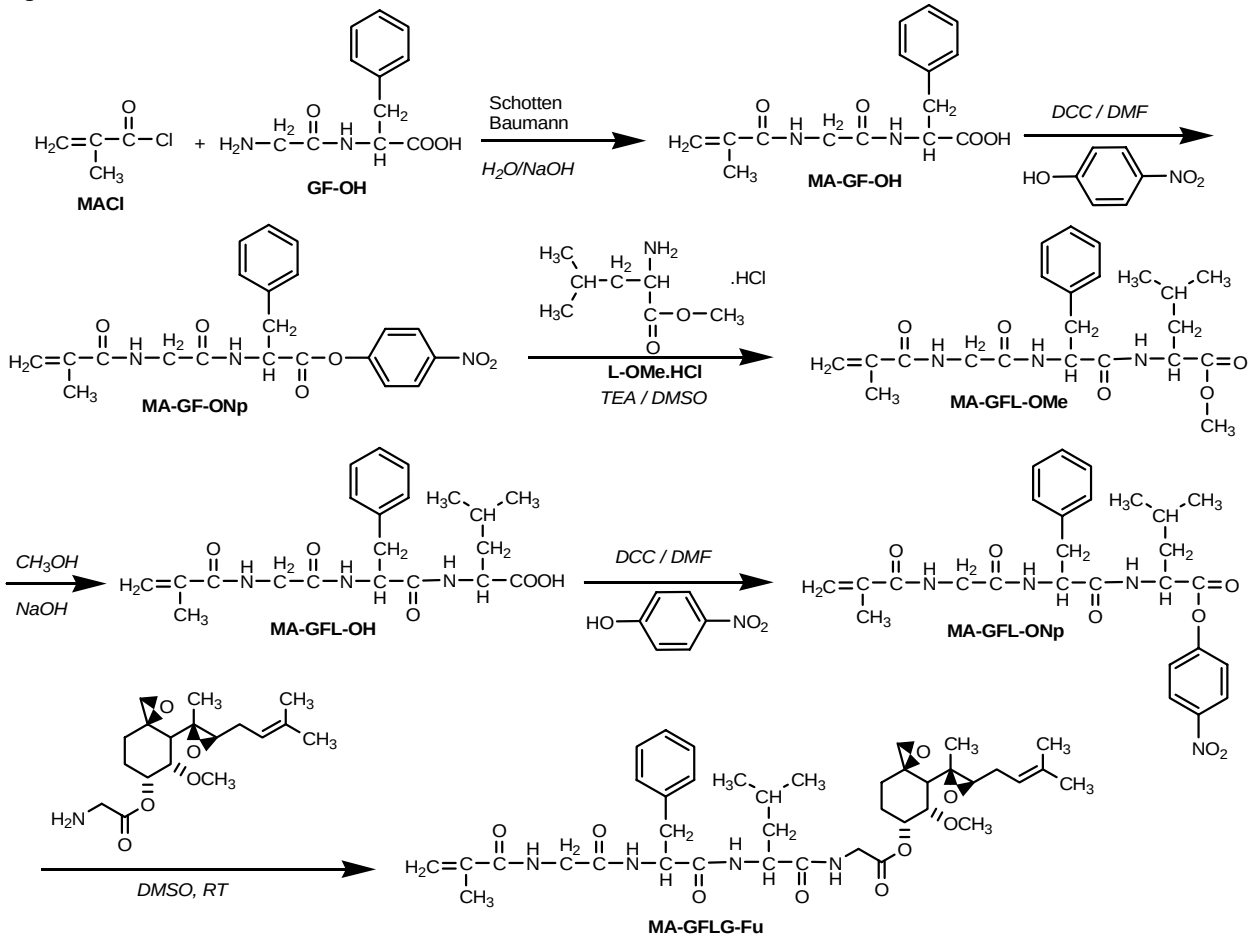

Figure 3. Scheme of synthesis of methacryloylglycylphenylalanylleucylglycyl-fumagillol (a polymerizable derivative of antiangiogenic drug Fumagillin with lysosomally degradable spacer).

Glycylphenylalanine (GF-OH) was dissolved in an equimolar solution of aqueous NaOH and cooled down to 0-5°C. An equimolar solution of methacryloyl chloride (MACl) in CH_2Cl_2 was added dropwise. Another equivalent of aqueous NaOH was subsequently added. The pH of the reaction was monitored to be around 8-9. After addition of MACl and NaOH, the reaction mixture was allowed to warm up to room temperature. The CH_2Cl_2 layer (containing unreacted MACl) was discarded. The combined aqueous phase was mixed with ethyl acetate (EtOAc) and acidified with dilute HCl to a pH around 2-3. The organic layer was separated and the aqueous layer further extracted with EtOAc. The combined organic layer was dried over Na_2SO_4, filtered and the filtrate rotavaped to obtain the product **(MA-GF-OH)**. Recrystallization was done from EtOAc (MW = 290.31).

To a solution of MA-GF-ONp in methylsulfoxide (DMSO), an equimolar solution of leucyl-methyl ester (L-OMe.HCl) in DMSO was added dropwise with constant stirring followed by a slight excess of triethylamine (TEA). The reaction mixture was stirred for 18 h at room temperature. The crude product namely methacryloylglycylphenylalanylleucyl-methyl ester **(MA-GFL-OMe)** was purified by precipitation from methanol in ether (MW = 417.50)

To a solution of MA-GFL-OMe in methanol cooled to 0°C, excess of 1(N) NaOH was added dropwise under stirring. The reaction mixture was stirred at 0°C for 1.5 h and at room temperature for 2 h. The reaction mixture was concentrated under vacuum to remove methanol, mixed with small amount of water and acidified with citric acid solution to pH 2.0. The free acid was extracted with EtOAc, washed with saturated brine and dried

over Na_2SO_4. The reaction mixture was subsequently concentrated under vacuum to obtain the product **(MA-GFL-OH)**. Recrystallization was done from EtOAc (MW = 403.47).

To a solution of MA-GFL-OH in DMF, an equimolar solution of p-nitrophenol was added with constant stirring. The temperature was cooled to -10°C and an equimolar solution of DCC in DMF was added dropwise with stirring. The reaction mixture was kept for 6 h at -10°C and at 4°C overnight. The precipitated DCU was filtered off and DMF removed by rotavaping. The crude product **(MA-GFL-ONp)** was purified by column chromatography (Silica Gel 60, Fluka, Germany), eluted with gradient of hexane and ethylacetate (1:0, 1:3, 1:1, and 0:1) (MW = 524.57)

To a solution of MAGFLONp in DMSO was added a 1.2 molar excess of Fu-G solution in DMSO with stirring. The crude mixture was purified from a methanol ether system to obtain **MA-GFLG-Fu** (MW = 724.9).

The final comonomer contains the drug Fumagillin attached to the polymerizable methacryloyl (MA) group via a well established lysosomally degradable tetra peptide spacer (GFLG). Compared to the previously synthesized comonomer in Year 1 of the grant, this compound contains one less glycine (G) unit in the spacer.

References:

1. Ruegg,C. *et al.* Evidence for the involvement of endothelial cell integrin alphaVbeta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma. *Nat. Med.* **4**, 408-414 (1998).